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A *hsp70* Gene-Based PCR for Detection of *Mycoplasma ovipneumoniae*

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Abstract: *Mycoplasma ovipneumoniae* is associated with atypical pneumonia in sheep and goats. Traditional detection of *M. ovipneumoniae* can be complex owing to its slow growth and fastidious nutritional requirements. In present study, researchers developed a PCR Assay Method for specific and sensitive detection of *M. ovipneumoniae* using primers targeting the heat shock protein 70 gene (*hsp70*). The results demonstrated that the *hsp70* gene-based PCR is specific by producing an amplicon of 135 bp only with *M. ovipneumoniae* strains. The detection limit is 4.4 pg which is 10 times more sensitive than the existing 16S rDNA-based PCR. The *hsp*70 gene-based PCR also demonstrated a higher detection rates for both nasal swabs and lung tissues when comparing with 16S rDNA-based PCR.

Key words: *Mycoplasma ovipneumoniae*, polymerase chain reaction, *hsp*70 gene, detection, China

INTRODUCTION

Mycoplasma ovipneumoniae is commonly isolated from sheep, goats and some wild ruminants in which it is believed to be associated with atypical pneumonia (Mohan *et al*., 1992; Besser *et al*., 2008; Rifatbegovic *et al*., 2011). While, *M. ovipneumoniae* can cause pneumonia in its own right, it can predispose the hosts to enhanced diseases from other pathogens such as *Mannheimia haemolytica* (Jones *et al*., 1982; Dassanayake *et al*., 2010).

Because of slow growth and fragile nature, traditional isolation and identification of *M. ovipneumoniae* are cumbersome and usually take 2 weeks. To improve the speed and accuracy of *M. ovipneumoniae* identification, a species-specific PCR targeting 16S rDNA was developed by Mcauliffe *et al*. (2003) and widely used in detection and epidemiological investigation of *M. ovipneumoniae* infection (Besser *et al*., 2008; Dassanayake *et al*., 2010; Ongor *et al*., 2011; Rifatbegovic *et al*., 2011). Except for the 16S rDNA-based PCR there is no other valid PCR Method for *M. ovipneumoniae* so far. This is probably due to low C+G content and high genomic heterogeneity as proved by the research to sequence whole genomes of type strain Y-98 and other 8 field strains, making it difficult to design an appropriate primer.

During a further study concerning heterogeneity following this initial sequencing study on *M. ovipneumoniae* strains, the *hsp70* gene (coding for heat shock protein 70) was found to be highly conserved among 15 *M. ovipneumoniae* strains with a sequence similarity of 96.1-100% and suitable as a target for PCR Method. Here in, researchers reported the development of a *hsp70* gene-based PCR for detection of *M. ovipneumoniae*.

MATERIALS AND METHODS

Bacterial strains and clinical samples: All bacterial strains used in this study are listed in Table 1. The *Mycoplasma* species were propagated in Hayflick broth containing 20% horse serum and other bacterial strains were grown in TSB (Trypticase Soy Broth) medium. Clinical samples include 98 nasal swabs and 28 lung tissues collected from 4 goat herds in Sichuan Province, China. All animals for sampling exhibited symptoms such as coughing, nasal discharge and dyspnea.

Primer: A primer pair (hsp70F: 5'-ACAACTCCTTCT GTTGTTGCCTT-3'; hspR: 5'-AGCACGAACAGTTTTAT CGCTAC-3') was designed based on an alignment of hsp70 sequences from 15 *Mycoplasma ovipneumoniae* strains. The primers amplify a highly conserved fragment of 135 bp in *hsp70* gene.

DNA preparation and PCR reaction: DNAs from bacterial cultures, nasal swabs and lung tissues were extracted using Standard Phenol/Chloroform Method. PCR was performed in a MyCycler™ Thermal Cycler (Bio-Rad,

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Table 1: Mycoplasma and bacterial strains tested with results of *hsp70* gene based PCR

Species	Strain	Source	*hsp70* gene-based PCR
M.ovipneumoniae	Y-98	CVCC[a]	+
M.ovipneumoniae	SC01	SWUN[b]	+
M.capricolum subsp. *capripneumoniae*	87001	CVCC	-
M. mycoides subsp. *mycoides* large colony	Y-goat	CVCC	-
M. mycoides subsp. *capri*	PG3	CVCC	-
M. agalactiae	PG2	CVCC	-
M.arginini	G230	CVCC	-
M. bovis	CD-2	SWUN	-
M. conjunctivae	QBJ-1	SWUN	-
M. hyopneumoniae	168	CVCC	-
Staphylococcus aureus	ATCC 6538	CVCC	-
Mannheimia haemolytica	1656	CVCC	-
Pasteurella multocida	SC-3	SWUN	-
Escherichia coli	013	SWUN	-

[a]China Veterinary Culture Collection Center; [b]Department of Veterinary Medicine, Sothwest University for Nationalities

USA). The 50 μL reaction volume contained 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 μM of each primer, 5 μL of 10×PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl), 1.25 units of Taq DNA polymerase (TaKaRa, Japan) and 2 μL of DNA template. An optimized PCR cycle profile included an initial denaturation step of 5 min at 95°C, followed by 35 cycles of 30 at 95, 30 at 60 and 30 sec at 72°C and a final extension step of 10 min at 72°C. PCR products were analyzed by electrophoresis on 1.5% (wt/vol) agarose gels containing ethidium bromide.

Specificity: In order to evaluate the specificity of the primer pair hsp70F/hsp70R, DNAs from several mycoplasma and bacterial species (Table 1) were examined. To verify the accuracy of amplicon, purified PCR product from strain Y-98 was cloned into pMD-T (TaKaRa, Japan) vector and confirmed by sequencing. To further test the specificity of the assay, a total of 36 *M. ovipneumoniae* genomic DNAs were tested by the *hsp70* gene-based PCR. These DNAs were extracted from *M. ovipneumoniae* field strains isolated from different geographical locations in China and are positive for the 16S rDNA-based PCR.

Sensitivity: To determine the sensitivity of the hsp70-based PCR and compare that to16S rDNA-based PCR, 10 fold serial dilutions of genomic DNA from *M. ovipneumoniae* Y-98 were prepared and tested using both hsp70-based PCR and 16S rDNA-based PCR.

Detection of clinical samples: A total of 98 nasal swabs and 28 lung tissues from goats with respiratory illness were tested for the presence of *M. ovipneumoniae* by both hsp70-based and 16S rDNA-based PCR.

RESULTS AND DISCUSSION

PCR specificity: As predicted, the PCR with primer pair hsp70F/hsp70R yielded a 135 bp amplicon. Sequencing for PCR product from *M. ovipneumoniae* Y-98 showed 100% identity to the *hsp70* gene sequence in GenBank (Accession No. HM047293.1). The *hsp70* gene-based PCR was positive for *M. ovipneumoniae* type strain Y-98 and field strain SC01. Other mycoplasma and bacteria species listed in Table 1 were negative by the PCR. The *hsp70* gene-based PCR could detect all 36 genomic DNAs which are positive for 16S rDNA-based PCR. This demonstrated that *hsp70* gene-based PCR developed in this study is specific for *M. ovipneumoniae*. This is helpful to discriminate *M. ovipneumoniae* from other *Mycoplasma* sp. and bacteria pathogens including *Mannheimia haemolytica*, *Pasteurella multocida* and members of the *Mycoplasma mycoides* cluster which are causative agents of respiratory illnesses in sheep and goats.

PCR sensitivity: Sensitivity of the assay was tested by amplifying 10 fold serial dilution of DNA of *M. ovipneumoniae* strain Y-98. The hsp70-based PCR could detect a minimum concentration of 4.4 pg DNA. As shown in Fig. 1 it is 10 times more sensitive than 16S rDNA-based PCR which exhibited a detection limit of 44 pg under the setting in present study. Sensitive detection of mycoplasmas is essential for the diagnosis of mycoplasma infections. The difficulties associated with the recovery and enrichment of these organisms from samples due to the fragileness and fastidious nutritional requirements of mycoplasmas make mycoplasma detection challenging. The increased sensitivity of this assay would enable detection of *M. ovipneumoniae* in samples with low mycoplasma loads. The sensitivity of the present PCR was also demonstrated by the significantly higher detection rate for both nasal swabs and lung tissues as described.

PCR test using clinical samples: The hsp70-based PCR gave positive results for 45.9% (45/98) nasal swabs and 67.9% (19/28) lung tissues while 16S rDNA-based PCR was positive for 38.8% (38/98) nasal swabs and 42.9%

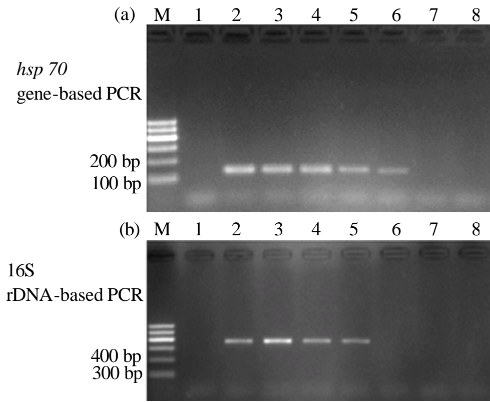

Fig. 1: Agarose gels showing the sensitivity of the a) *hsp70* gene-based PCR assay and b) 16S rDNA-based PCR using 10 fold serial dilutions of the genomic DNA of *M. ovipneumoniae* Y-98. Lane M: DNA molecular weight standard; Lane 1: Negative control without target DNA; Lane 2-8: 4.4 ng to 44 fg of genomic DNA in PCR reaction mixture

(12/28) lung tissues. Thus, this newly developed PCR assay might have higher clinical sensitivity than previously described 16S rRNA PCR technique might.

CONCLUSION

The hsp70-based PCR assay demonstrated high specificity and increased sensitivity and could have applications in clinical diagnostics and epidemiological studies.

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